Genetic differentiation, clinal variation and phenotypic associations with growth cessation across the *Populus tremula* photoperiodic pathway

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Running head: Genetic differentiation in the photoperiodic pathway of *P. tremula*
Abstract

Perennial plants monitor seasonal changes through changes in environmental conditions such as the quantity and quality of light. To ensure a correct initiation of critical developmental processes, such as the initiation and cessation of growth plants have adapted to spatially variable light regime and genes in the photoperiodic pathway have been implicated as likely sources for these adaptations. Here we examine genetic variation in genes from the photoperiodic pathway in *Populus tremula* (Salicaceae) for signatures diversifying selection in response to varying light regimes across a latitudinal gradient. We fail to identify any loci with unusually high levels of genetic differentiation among populations despite identifying four SNPs which show significant allele frequency clines with latitude. We do however observe large covariance in allelic effects across populations for growth cessation, a highly adaptive trait in *P. tremula*. High covariances in allelic effects is a signature compatible with diversifying selection along an environmental gradient. We also observe significantly higher heterogeneity in genetic differentiation among SNPs from the photoperiod genes than among SNPs from randomly chosen genes. This suggest that spatially variable selection could be affecting genes from the photoperiod pathway even if selection is not strong enough to cause individual loci to be identified as outliers. SNPs from three genes in the photoperiod pathway (*PHYB2, LHY1* and *LHY2*) show significant associations with natural variation in growth cessation. Collectively these SNPs explain 10-15% of the phenotypic variation in growth cessation. Covariances in allelic effects across populations help explain an additional 5-7% of the phenotypic variation in growth cessation.
**Introduction**

Species occupying heterogeneous environments are often subjected to spatially variable natural selection, that is, natural selection that differs among populations that a species inhabit. Such spatially variable selection is expected to have important consequences for how phenotypic variation is partitioned within and among populations and also for genetic differentiation at the underlying loci controlling these traits (Latta, 1998, Le Corre and Kremer, 2003, Whitlock, 2008). A major motivation for studying the genetic consequences of local adaptation is to identify loci that control local adaptation. The identification of loci (and traits) subjected to spatially variable selection is complicated by the fact that genetic drift introduce heterogeneity among loci. However, it should be possible to identify putative targets of spatially variable selection by searching for loci with unusually high levels of genetic differentiation (Lewontin and Krakauer, 1973, Beaumont 2005). Although the initial idea championed by Lewontin and Krakauer (1973) have attracted much criticism, the general principle of searching for loci with unusually high levels of genetic differentiation has proved to be quite successful in identifying loci subject to spatially variable selection, especially when selection is strong relative to migration (eg. Beaumont and Balding, 2004, Beaumont 2005).

However, even though spatially variable selection acting on a quantitative trait conferring local adaptation may be strong, selection on the underlying causal loci can often be quite weak because individual loci only contribute a small proportion to the total phenotypic variation (Latta, 1998, Le Corre and Kremer, 2003). In such cases migration may overwhelm local selection acting on the underlying QTLs (that is, \( m > s \)) and genetic differentiation at these loci is actually well approximated by genetic differentiation at neutral loci, even though genetic differentiation at the quantitative traits themselves can be high (Latta, 1998, Whitlock, 2002, Le Corre and Kremer, 2003). The reason for this is that selection generates linkage disequilibrium between loci and the
combined effect of multiple loci changing in parallel is much greater than what can be predicted from the effects of individual loci alone (Latta, 1998, Le Corre and Kremer, 2003). This potentially limits the utility of $F_{st}$-based outlier detection methods for identifying loci controlling quantitative traits involved in mediating local adaptation.

A classic example of local adaptation in the face of substantial gene flow is adaptation to local photoperiod seen in many boreal forest trees (Chen et al. 2002). Trees alternate between growth when conditions allow and dormancy during periods of unfavorable environmental conditions and a correct timing of growth initiation and growth cessation represents a crucial trade off between growth and survival. In boreal trees the onset of winter dormancy is largely determined by seasonal changes in photoperiod (Chen et al. 2002) and genes in the photoperiodic pathway have been implicated in the control of growth cessation (Frewen et al. 2000, Böhlenius et al. 2006; Gyllenstrand et al. 2007, Ingvarsson et al. 2008). The photoperiodic pathway has been dissected in great detail in the model plant *Arabidiopsis thaliana* and this have resulted both in the identification of individual genes making up the pathway and the interactions among these genes (Eriksson and Millar 2003, Hayama and Coupland 2003). Although distinct photoperiodic responses appear to have evolved independently several times in the evolutionary history of plants, the photoperiodic pathway is conserved between monocots and dicots (Cremer and Coupland 2003) and even between angiosperms and gymnosperms (Gyllenstrand et al. 2007).

Ingvarsson et al. (2008) recently showed that two mutations in the phytochrome photoreceptor *PHYB2* are independently associated with naturally occurring variation in the timing of bud set or growth cessation European aspen (*Populus tremula*, Salicaceae). However, these mutations explain only a few percent of the observed phenotypic variation in growth cessation. As this trait appear to be under tight genetic control with little environmental influence in *P. tremula* (Hall et al. 2007; Luquez et al. 2008), other genes besides *PHYB2* must be involved in mediating natural variation in growth cessation. Here we use a candidate gene approach to search for signs of local adaptation and
phenotypic associations to growth cessation in genes from the photoperiod pathway. Our goal is to identify loci involved in controlling natural variation in growth cessation and to study to what degree genes from the photoperiodic pathway show evidence for divergent natural selection. Finally, we test the predictions from Latta (1998) and Le Corre and Kremer (2003) of how divergent selection on adaptive traits influence genetic differentiation at the underlying QTLs.

**Material and Methods**

*PCR amplification and DNA sequencing*

All plant material used in this study were selected from the Swedish Aspen Collection (the SwAsp collection) which consists of 120 trees collected from 12 sites throughout Sweden (ten trees per population) along a latitudinal gradient spanning approximately ten latitude degrees. The SwAsp collection is described in more detail in Hall et al. (2007) and Luquez et al. (2008).

Total genomic DNA was extracted from frozen leaf tissue using the DNeasy plant mini prep kit (Qiagen Inc. Valencia, CA). Sequences for 25 genes from the photoperiod pathway were taken from Hall et al. (unpubl.). Briefly, for a subset of 13 genes all exons (excluding short exons, <80bp) were sequenced directly from PCR amplified fragments from genomic DNA. For these genes we sequenced 24 individuals. The remaining 12 genes were PCR amplified from 12 individuals and cloned into the pCR2.1 vector using a TA-cloning kit from Invitrogen (Carlsbad, CA). All fragments were sequenced on a Beckman CEQ 2000 capillary sequencer at the Umeå Plant Science Centre sequencing facility. Sequences were verified manually and contigs were assembled using the computer program Sequencer v 4.0 (Gene Codes Corporation, Ann Arbor, MI). For genes that were directly sequenced, polymorphic sites were directly visible from double peaks in the chromatograms generated by the sequencer. For cloned genes, four or more colonies for each individual were sequenced in an attempt to identify the two haplotypes present within an individual and to control for Taq polymerase transcription errors. We also obtained the homologous gene
regions from the *Populus trichocarpa* genome sequence and used these as outgroup sequences to polarize mutations in to ancestral and derived states based on the allelic state in *P. trichocarpa*.

We selected all SNPs from these genes that had a frequency greater than 15% in the sample of sequences we obtained. We developed proofreading allele-specific extension assays (PRASE) for these SNPs (Di Gusto and King 2004) and they were scored from the complete SwAsp collection (Hall et al. 2007, Luquez et al. 2008). Using these criteria we managed to score a total of 110 SNPs from 23 out of the 25 genes, for an average of 4.8 SNPs per gene (minimum 1, maximum 14, see Supplementary Table 1 for information on the number of SNPs scored per gene). In addition we included the three most common alleles from a short, tri-nucleotide microsatellite located in the coding region of the *COL2B* gene (Hall et al. 2007). Alleles at this microsatellite show variable numbers of poly-E (Glu) repeats with repeat numbers ranging from five to ten Glu amino acids (St Onge 2006, Hall et al. 2007). These alleles were coded as “pseudo-SNPs”, where the presence of the SSR allele was coded as 0, 1 or 2, depending on the number of copies of that allele an individual carried. Two low-frequency alleles (frequencies 0.5%, 1.3%) at the *COL2B* microsatellite were not included in the analyses. For two genes (*TOC1* and *COL2A*) no SNP passed our frequency criteria to be included in our scoring assay.

We also scored 93 SNPs from 21 genes that are not thought to be involved in the photoperiodic control of phenology. These genes were taken from the 77 genes analyzed in Ingvarsson (2008) and SNPs were selected using the same criteria that was use to select SNPs from the photoperiod genes. For brevity we refer to the first set of SNPs as “photoperiod SNPs” and the latter as “control SNPs” throughout this paper.

**Data analyses**

Genetic diversity was estimated for each population by calculating observed ($H_o$) and expected ($H_e$) heterozygosities. Tests for Hardy-Weinberg equilibrium were performed for each SNP using
the genetics package in R (R Development Core Team 2008). This package was also used to test for pairwise linkage disequilibrium between individual SNPs using a maximum likelihood approach as the genotype data is unphased and individual haplotypes can not be distinguished. We also tested for clinal variation in SNP frequencies by regressing population allele frequencies on latitude of origin.

Genetic differentiation among populations for individual SNPs was estimated using the Bayesian method of Foll and Gaggiotti (2008). This method models allele frequency counts at a specific locus in a population using a multinomial-Dirichlet distribution which captures the underlying genealogical structure of the migration process among populations (Foll and Gaggiotti 2008, see also Beaumont and Balding 2004). This method allows for the estimation of both locus-specific effects ($\alpha_i$), population-specific effects ($\beta_j$) on genetic differentiation and these effects are estimated using logistic regression and subsequently used to calculate locus-specific estimates of $F_{ST}$. In the method of Foll and Gaggiotti (2008) the effects of selection at a locus is evaluated by fitting two alternative models to the data. One model (M0) only includes population specific effects ($\beta_i$) whereas the alternative model (M1) includes both population and locus specific effects ($\alpha_i$ and $\beta_i$). These models are compared using their respective posterior probabilities by calculating the Bayes Factor (BF) which is the ratio of posterior probabilities for the two alternative models (that is \( BF = P(M1|D)/P(M0|D) \), Foll and Gaggiotti [2008]). We analyzed the SNP data using the BayesScan program that implements the method of Foll and Gaggiotti (2008) (available at http://www-leca.ujf-grenoble.fr/logiciels.htm). Since this analysis is clearly exploratory and is aimed at identifying possible loci under diversifying selection we used a relatively modest threshold for identifying outliers. Foll and Gaggiotti (2008) showed that bi-allelic markers, like SNPs, have lower power to detect selection than multi-allelic markers (eg. SSRs) and we therefore used a rather liberal cut-off limit for regarding loci as potential outliers. In practice we used a posterior probability for the selection model (M1) of 86% (corresponding to a log_{10}(Bayes Factor) of 0.79) to identify potential
outlier loci. Simulations have shown that for SNPs this cut-off limit yields an approximate 5% false 
discovery rate (Foll and Gaggiotti 2008).

$F_{ST}$ values for QTLs are expected to show greater heterogeneity than putative neutral loci (Le 
Corre and Kremer 2003) and to test this hypothesis we compared the heterogeneity of $F_{ST}$ values 
between the photoperiod SNPs and the control SNPs using the method of Lewontin and Krakauer 
(1973). Among-locus heterogeneity, $k$, was calculated as:

$$k = \frac{(d - 1) V(F_{ST})}{E(F_{ST})^2}$$

(1)

where $d$ is the number of populations and where $E$ and $V$ denote expectation and variance, 
respectively. For neutral loci, $k$ is expected to be close to 2 (Lewontin and Krakauer 1973). We 
tested the differences in heterogeneity in $F_{ST}$ values between photoperiod and control SNPs in two 
ways. First we compared the variance in $F_{ST}$ between the two groups of SNPs using Bartlett's test of 
homogeneity of variances. Second, we used a resampling test to account for the fact that the number 
of SNPs differ between the two groups. We generated the null-distribution of $k$ by randomly 
assigning SNPs (without replacement) to the candidate gene and control groups and calculating $k$ 
using Equation (1) for these two groups. This procedure was repeated $10^4$ times and the observed 
value of $k$ was compared to this distribution.

Theory suggest that in species experiencing diversifying selection in the face of high levels of 
gene flow, positive covariance (i.e. linkage disequilibrium) may build up across loci, so that even 
when allele frequencies changes are relatively modest across populations, the combined effect of 
many loci, changing in parallel, can be substantial for quantitative traits controlled by these loci 
(Latta 1998, Le Corre and Kremer 2003). We therefore estimated the covariance of allele 
 frequencies across populations for all pairwise combinations of SNPs. The covariance of allelic 
effects is

$$4a_ia_jD_{ij},$$

(2)
where $a_i$ and $a_j$ are the additive effects of locus $i$ and $j$, respectively and $D_{ij}$ is the linkage disequilibrium between the two loci (Latta 1998). As shown by Ohta (1982, see also Latta 1998, Le Corre and Kremer 2003) total linkage disequilibrium can be partitioned into within and between-population components where the between-population component of linkage disequilibrium is given by:

$$D_{b(ij)} = p_i p_j^* - p_i^* p_j$$

This method is virtually identical to that used in Storz and Kelly (2008, their Equation 3) with the exception that their estimator is standardized with respect to allele frequencies and is squared to remove the sign of the LD. Since we are specifically interested in loci showing positive covariance across populations (Latta 1998) we choose preserve the sign in our calculations. Also, to avoid inflated covariances between SNPs because of intragenic LD, we only performed pairwise comparisons between SNPs from different genes. The additive effects of locus $i$ and $j$ in Equation (1) were calculated from estimated from our association analyses (see below).

We calculated the covariance of allelic effects across populations for all SNP pairs using Equation (2) for both control and photoperiod SNPs using additive effects ($a$) estimated from data on growth cessation in these populations (Ingvarsson et al. 2008). To explore the expected distribution of the covariance among SNPs we simulated 113 SNPs using the approach of Nicholson et al. (2002). Briefly, this method simulates multi-population unlinked genotype data from a multinomial Dirichlet model (see Nicholson et al. 2002 for more details). The simulations were tailored to give an expected value of $F_{ST}$ equal to the average value of $F_{ST}$ we observed in our sample of SNPs. All simulations were performed using the *simMD* function from the *popgen* package in *R*. The simulated SNPs were then used to calculate additive effects from the growth cessation data and subsequently to calculate the covariance among allelic effects using Equation (2). The simulated SNP data was used to estimate the expected distribution of covariance of allelic effects under no diversifying selection.
Phenotypic associations

We used single marker association tests assuming a model of additive effects of alleles within a marker locus. To control for possible spurious associations caused by genetic structuring of the sample, we used the mixed-model method of Yu et al. (2006) which allows for both population structure and more diffuse familial structure within the sample. Population structure and pairwise kinship coefficients were estimated using 26 putatively neutral SSR markers using the program Structure (Prichard et al. 2000) to estimate population membership for each individual using K=2 (Ingvarsson et al. 2008). Similarly, the matrix of pairwise kinship coefficients was calculated according to Ritland (1996) using the software package SpaGeDi (Hardy and Vekemans 2002). The mixed-model we used was:

\[ y = X\beta + Zu + \epsilon \]

where \( y \) is a vector of observed phenotypes, \( \beta \) is a vector on parameters for fixed effects (including SNP effects) and \( u \) and \( \epsilon \) are vectors that capture (random) variation due to unmeasured polygenic contributions (\( u \)) or environmental effects (\( \epsilon \)). \( X \) and \( Z \) are design matrices relating fixed and random effects to individual observations. The variance of polygenic (clone) effects equals \( V(u) = 2KV_G \), where \( K \) is the matrix of kinship coefficients. Finally, the variance of the residual effects is \( V(\epsilon) = RV_R \), where \( R \) is a matrix with zeros for all off-diagonal elements and the reciprocal of the number of observations along the diagonal. \( V_G \) and \( V_R \) measure the genetic and residual variances for the trait.

We combined phenotypic data on growth cessation from our two common garden sites (Luquez et al. 2008) across all years of our survey (2004-2008). We therefore included fixed effects for block, site and year in the mixed-model, in addition to variables included to control for population structure into the vector of fixed effects (\( \beta \)). We implemented our association analyses using the kinship library in R, and fitted the model using restricted maximum likelihood methods. We used
the false discovery rate (FDR, Storey and Tibshirani 2003) to control for multiple testing across the 206 SNPs.

**Results**

**Genetic differentiation at SNPs**

We analyzed 113 SNPs from 23 genes in the photoperiod pathway and 93 SNPs from 21 control genes. We could not reject Hardy-Weinberg equilibrium within populations for either photoperiod or control SNPs (data not shown). However, the power of these tests is quite low with only ten individuals sampled per population. For the pooled samples, across populations, 19 SNPs from the photoperiodic pathway genes show significant deviations from Hardy-Weinberg expectations after multiple-test correction. All but two of these show a slight excess of heterozygotes across the twelve populations. For the 93 SNPs from the control loci we found 2 SNPs where we could reject Hardy-Weinberg equilibrium and both show an excess of homozygotes across populations. We can not rule out that these minor deviations observed are caused by experimental artifacts. The PRASE assay is highly specific and we do not expect that misamplification of the alternate allele in each SNP pair is a contributing factor (DiGusto and King 2004). A possible experimental error would be failed amplification due to polymorphisms in the primer binding sites. This would result in either failed amplification from some individuals or an excess of homozygotes if amplification failed in heterozygous individuals. We observe neither of these patterns and in fact we observe an excess of heterozygotes suggesting that PCR amplification errors are not causing the slight deviations from Hardy-Weinberg we observe in our data set. In line with earlier results (Ingvarsson et al. 2008, Ingvarsson 2008) we find relatively little LD between SNPs. A total of 139 out of 1741 pairwise comparisons (7.9%) between SNPs showed evidence for significant LD after multiple-test corrections. However, we do not observe any clear physical clustering of sites in LD and sites in close physical proximity often showed negligible levels of LD (data not shown).
We detect four SNPs where allele frequencies vary with latitude after multiple-test correction using a false discovery rate of 0.1 (Figure 1). Two of these SNPs, PHYB 1823 (T608N) and PHYB2 6147 (Y1117F) from the phytochrome gene PHYB2, have already been shown to exhibit clinal variation in earlier studies (Ingvarsson et al. 2006). In this study we have identified two additional SNPs showing significant clinal variation. These two SNPs are located in the circadian clock genes LHY1 and LHY2, respectively. The LHY1 1466 mutation is an A-to-T transversion that results in a synonymous mutation located in exon 4 of the LHY1 gene. The derived allele (T) is most common in northern populations, increasing from a frequency of around 10% in the southern most population to around 40% in the northern-most population. The LHY2 2327 SNP is a G-to-A transition in exon 6 of LHY2 resulting in Ser → Asn replacement (S681N) in the translated protein sequence. For the LHY2 mutation, the derived allele (A) is most frequent in the southern populations and is virtually absent in the northernmost populations. This contrasts with mutations showing clinal variation in PHYB2 (Ingvarsson et al. 2006) and LHY1 where the derived allele increase in frequency with latitude. Interestingly, the LHY2 SNP shows a third allelic state (C) in P. nigra which results in the incorporation of a Thr amino acid into the LHY2 protein (Takata et al. 2009).

The average genetic differentiation across populations (mean $F_{ST}$) over all 206 SNPs was 0.017 which very close to estimates of $F_{ST}$ based on microsatellite data ($F_{ST}$= 0.015, Hall et al. 2007). There was also no significant difference in mean $F_{ST}$ between photoperiod and control SNPs (0.018 and 0.016, respectively, Wilcoxon two sample test, $p=0.33$). We failed to identify any SNPs with significantly enhanced genetic differentiation due to the action of natural selection (Figure 2). It is interesting to observe that none of the four loci where we demonstrate significant clinal variation are identified as outliers. In fact, these four loci have $F_{ST}$ values that fall right in the middle of the distribution of $F_{ST}$ values (Figure 2).

We quantified heterogeneity in $F_{ST}$ values among loci for the photoperiod and control SNPs.
respectively using the approach outlined by Lewontin and Krakauer (1973). We find evidence for substantially greater heterogeneity in genetic differentiation among photoperiod SNPs (k=4.27) than among control SNPs (k=2.03) and this difference is significant using either a parametric test (Bartlett's test of homogeneity of variances, $K^2=11.7$, $df=1$, $p<0.001$) or using a permutation test to account for differences in the number of loci in the two sets of SNPs ($p=0.025$, Figure 2C). Since estimates of $F_{ST}$ are intimately tied to expected heterozygosity (Beaumont and Nichols 1996) we also tested whether the heterozygosity differed between the two classes of SNPs to ensure that the differences in $F_{ST}$ we observe are not simply caused by differences in heterozygosity at the underlying loci (Figure 2B). However, we found no significant difference in either the expected heterozygosity ($H_T$) between photoperiod and control SNPs ($t=0.498$, $df=60$, $p=0.62$) or in the heterogeneity of heterozygosity values between the two classes (Bartlett's test of homogeneity of variances, $K^2=0.344$, $df=1$, $p=0.558$). This demonstrates that the increased heterogeneity in genetic differentiation we observe in photoperiod SNPs is not caused by differences in genetic diversity between SNPs from the two classes of genes.

In the absence of diversifying selection, simulations suggest that among-population LD should generally be low. The data from the control SNPs also show a good correspondence with the simulated data. In the SNPs from the photoperiod pathway, however, we observe a substantial number of locus pairs that show unusually large (positive and negative) covariances of allelic effects, when compared to either simulated values or to control SNPs (Figure 3). A total of 239 SNP pairs from the photoperiod pathway (5.5%) have covariances that fall outside of the 99% confidence interval calculated from the reference SNPs. Interestingly, of the five SNP pairs that show the highest covariance in allelic effects across populations, all but one involve at least one SNP showing significant clinal variation (Table 1).

**Phenotypic associations**

After correcting for multiple testing, six SNPs show significant associations with growth
cessation (Table 2). Two of these SNPs are the non-synonymous mutations (T608N and L1078P) located in the PHYB2 gene that we have previously shown to be significantly associated with growth cessation in P. tremula (Ingvarsson et al. 2008). In addition to the two PHYB2 SNPs we have identified an additional SNP in PHYB2, S383T, that is also associated with onset of growth cessation in P. tremula. Furthermore, two SNPs in the circadian clock genes LHY1 (syn1466) and LHY2 (S681N), also that show significant association with natural variation in growth cessation. Like the T608N SNP in PHYB2, the LHY1 and LHY2 SNPs both show significant allele frequency clines with latitude. The two mutations explain 6.7% and 12.7% of the observed phenotypic variation in growth cessation (Table 2, Figure 4), although these estimates are likely overestimating the true effect sizes by two to three-fold (Ingvarsson et al. 2008, see also Discussion below).

Finally, one allele at the coding polyE-repeat SSR in COL2B, E9, is also associated with growth cessation.

We included all SNPs into a single model to test for the effect of each SNPs individually while statistically controlling for variation at the other putative causal SNPs. This analysis shows that all SNPs, except the polyE-repeat allele at COL2B, are independently associated with growth cessation (Table 2). Together the five SNPs that remain significant in the full model explain almost 33% of the phenotypic variation in growth cessation. To further take into account the positive covariances we observe among SNPs we also analyzed a model including the five SNPs and their pairwise interactions and with this model the amount of variation explained increase to 42.5%. Again, we expect that these effects are overestimating the true effects of the SNPs due to the small size of our association mapping population.

Discussion

Clines and detection of outlier loci

In addition to the SNPs in PHYB2 were we already have documented allele frequency clines
Ingvarsson et al. (2006), we have identified two additional SNPs showing strong clinal variation with latitude, one each in the two circadian clock-associated genes *LHY1* and *LHY2* (Figure 1). The presence of such allele frequency clines is a strong sign of spatially variable selection acting directly at or near these mutations (Barton 1999). Two possible factors that are relevant for our identification of allele frequency clines in *P. tremula* are worth mentioning here. First, we have only sampled ten individuals per population and the rather low number of individuals can have a substantial effect on the estimation of allele frequencies. However, such sampling noise likely acts to reduce our power to detect allele frequency clines. Second, Swedish populations of *P. tremula* show evidence for recent admixture between lineages originating from different glacial refugia (De Carvahlo et al. 2010). The clines we observe could therefore be a product of gene flow between two partly divergent populations (De Carvahlo et al. 2010), a view that is further supported by the presence of weak, but significant, isolation by distance we found in our study populations based on microsatellite data (Hall et al. 2007). We do not, however, observe any isolation by distance in the SNP data presented here (Mantel test, $p=0.413$). Furthermore, if clines where the product of admixture we would expect a substantially greater number of loci to show clinal variation. The fact that we only observe four loci suggest that clinal variation is not common in *P. tremula* and these loci are hence more likely the product of spatially variable selection rather than admixture. De Carvahlo et al. (2010) also showed that the cline for growth cessation in *P. tremula* is much steeper than linear at the center of the cline and the data is better described by a step-cline. Steep phenotypic clines are expected on theoretical grounds (Barton 1999), but it is not clear whether also clines at the underlying loci are expected to be steeper than linear. To explore this, we fitted the non-linear model from De Carvahlo et al. (2010), which allows for a steeper slope in the center of the cline, to the individual SNPs showing allele frequency clines (Figure 1). However, in all cases allele frequency clines were best described by a simple linear change in allele frequency with latitude (data not shown), consistent with much weaker selection acting on individual QTLs (Latta
Theory predicts that when a quantitative trait is subject to diversifying selection in the face of substantial migration, average levels of genetic differentiation at individual loci controlling quantitative trait variation (QTLs) can be hard to distinguish from genetic differentiation at completely neutral loci (Latta 1999, Whitlock 2002, Le Corre and Kremer 2003), suggesting that it might be difficult to identify QTLs as outlier loci with unusually high levels of genetic differentiation. In accordance with this prediction we do not find any loci that show unexpectedly high levels of population structure among either the photoperiod or control loci (Figure 2). Perhaps surprisingly this also applies to the four SNPs for which we demonstrate significant allele frequency clines and which hence are clear candidates for being influenced by spatially variable selection. The near complete lack of outlier loci can, at least partly, be ascribed to an ascertainment effect since we purposely selected SNPs that were relatively common across the sample range and which therefore are perhaps less likely to show large frequency differences between populations.

A growing number of studies that have identified outlier loci exhibiting unusually high levels of genetic differentiation among populations using genome-wide approaches (see for instance Nosil et al. 2009, for a recent review), while relatively few studies have taken a more targeted candidate gene approach which includes loci with known or suspected effects on the trait conferring local adaptation. Our results suggest that a genome-wide scan for loci with higher than expected $F_{ST}$ values would have failed to identify all four SNPs that nevertheless show clinal variation and hence strong evidence for spatially variable selection. We can think of two possible reasons for this; either is our data from *P. tremula* atypical in some way or genome-wide approaches will only identify a subset of loci subjected to divergent selection. In this respect, allele-frequency clines provide substantially stronger evidence for diversifying selection at, or near, these SNPs than patterns of genetic differentiation among populations.
Heterogeneity in levels of genetic differentiation

Gene flow among populations in *P. tremula* is very high (genetic differentiation at neutral markers is $F_{ST} = 0.018$, Hall et al. [2007], the mean $F_{ST}$ for the SNPs in this study is 0.017), and it is likely that the effects of migration overwhelms selection acting on individual causal SNPs and thus limits our ability to detect a signal of diversifying selection by yielding loci with unusually high levels of genetic differentiation. One observation supporting this interpretation is the significantly higher heterogeneity we observe among $F_{ST}$ values for the photoperiod SNPs ($k = 4.27$) compared to control SNPs ($k = 2.03$, see also Figure 3). Thus, even though selection is not strong enough to generate $F_{ST}$-values that are different enough from the background level of genetic differentiation to be classified as outliers, the variance in $F_{ST}$ among QTLs is higher than at putative neutral markers (Le Corre and Kremer 2003, Whitlock 2008). This suggests that diversifying selection is acting on at least a few of the photoperiod SNPs, but that selection is not strong enough to result in the identification of clear outlier loci using $F_{ST}$-outlier methods (Le Corre and Kremer 2003).

Parallel clines and the covariance of allelic effects

Under strong diversifying selection and high levels of gene flow, theory predicts that population differences in the mean of a quantitative trait will partly be caused by positive covariances (that is, linkage disequilibrium) that build up between individuals QTLs (Latta 1999, Le Corre and Kremer 2003). We can also confirm this observation in our data, where several pairs of loci shows what appear to be unusually large co-variation of allelic effects on growth cessation across the latitudinal cline (Figure 3, Table 1). As predicted by theory, at least one locus, and in some cases both loci, from the five SNP pairs displaying the highest covariances also show significant latitudinal allele frequency clines (Table 1). For instance, the locus pair with the highest observed covariance consists of the S681N SNP from *LHY2* and the T1078P SNP from *PHYB2* (Table 1). The covariance of allelic effects of the S681N/T1078P SNP-pair is roughly 25 standard deviations greater than the mean covariance seen among reference SNPs.
A similar approach to what we have used here to study the effects of spatially divergent selection on allelic associations across populations was recently proposed by Storz and Kelly (2008). Storz and Kelly (2008) showed that spatially variable selection among altitudinal populations of deer mice (*Peromyscus maniculatus*), driven by differences in the partial oxygen pressure at low and high-altitude populations, results in strong between-population linkage disequilibrium at two α-globins. These α-globins have previously been shown to contribute to differences in aerobic capacity between mice from low or high-altitude populations and are hence likely candidate genes mediating local adaptation to variation in oxygen pressure (Storz et al. 2007). They also showed that while genetic differentiation between functionally distinct allelic classes at the two α-globin genes was strong, genetic differentiation between populations was not exceptionally high, presumably due to high levels of gene flow between populations (Storz and Kelly 2008). Storz and Kelly (2008) also concluded that casual substitutions would not have been identified based on patterns of genetic differentiation alone, a pattern similar to what we observe in *P. tremula* (see above).

**Phenotypic associations in the photoperiod pathway**

We identified five SNPs, three in *PHYB2* and one each in *LHY1* and *LHY2*, that are independently associated with naturally occurring variation in growth cessation (Table 2, Figure 4). Individually these SNPs explain between 6 and 12% of the observed genotypic variation in growth cessation and together the total variation explained is over 30%. The naïve estimation of the variation explained are likely overestimating the true effects of the mutations at least by two to three-fold, since these effects are estimated from a relatively small mapping population. In a previous study we used an *ad hoc* method (Allison et al. 2002) to show that the phenotypic effects of mutations are overestimated by two to three-fold (Ingvarsson et al. 2008). However, even if we take this into account, the five mutations likely explain 10-15% of the naturally occurring variation in growth cessation. Furthermore, this only measure the amount of variation explained by allelic
variation at these five SNPs. As discussed above, theory predicts that a large fraction of the observed phenotypic variation is actually explained positive covariances among causal SNPs that builds up as allele frequencies change in parallel across populations (Latta 1998, Le Corre and Kremer 2003). Taking covariances between loci into account increase the variation explained to 42.5% which corresponds 14-21% after correcting for overestimation. This shows that 20-25% of the phenotypic variation observed in growth cessation can not be attributed directly to individual SNPs but is rather explained by covariances among among individual causal mutations. Such covariances could thus make an important contribution to the proportion of variation explained in many quantitative traits and partly explain why the amount of variation explain is usually far below that predicted based heritabilities of the traits, a phenomenon highlighted in the human-genetics community as the “missing heritability problem” (Manolio et al. 2009).

Another potential bias affecting the amount of variation explained is that the SNPs we have identified may not be causal but are in fact only in imperfect LD with the true causal SNP. These true causal SNPs can either be segregating at a lower frequency or in occur in an unsampled region of the gene (eg. regulatory regions in introns of UTs) and in such cases true QTL effect of the gene can be substantially larger than what we detected using the sampled SNPs. Given the low levels of LD observed in P. tremula, extending only a few hundred bps (Ingvarsson 2005, Ingvarsson 2008), we believe this is unlikely and we are reasonably confident that the SNPs we have identified are the causal mutations. However, all association will necessarily have to be verified in either an independent mapping population or using detailed functional studies.

Phytochrome signaling is known to be mediated through physical interactions between the Pfr form of the phytochrome protein and transcriptional regulators that are constitutively present in the nuclei before light signal perception (Martinez-Garcia et al. 2000). Interestingly, the phytochrome interacting protein PIF3 has been shown to bind to elements in the LHY promoters and PHYB then interacts with the promoter bound PIF3, thereby inducing LHY expression (Martinez-Garcia et al.
In view of these possible interactions between the \textit{PHYB} and \textit{LHY} genes it is interesting to note that the by far strongest covariance in effects across populations we observe among SNPs is between the L1078P SNP in \textit{PHYB2} and the S681N SNP in \textit{LHY2}. The \textit{PHYB2} SNP (L1078P) occurs in a region of the \textit{PHY} protein with poorly characterized function but that is possibly involved in mediating downstream signaling (Chen et al. 2004). The residue in \textit{LHY2} harboring the mutation that is significantly associated with growth cessation in \textit{P. tremula} is highly conserved among other angiosperms. Interestingly, the same residue is segregating yet another amino acid in the closely related species \textit{P. nigra}, and the mutation responsible for this amino acid substitution occurs at the same site that is polymorphic in \textit{P. tremula}, although \textit{P. nigra} harbors a third allelic state at this site (Takata et al. 2009).

The \textit{LHY} protein itself is a core component of the central oscillator of the circadian clock and is crucial for sustaining circadian rhythms in plants (Alabadi et al. 2001). In \textit{Arabidopsis} and rice two homologous genes, \textit{LHY} and \textit{CCA1}, together with \textit{TOC1} make up the central oscillator of the circadian clock (Hayama and Coupland 2004, Murakami et al. 2006). The \textit{Arabidopsis} \textit{LHY} and \textit{CCA1} genes have partially redundant function but both are crucial for maintaining sustained circadian rhythms (Alabadi et al. 2001). Interestingly, \textit{Populus} lacks a homolog of the \textit{CCA1} gene and it is possible that the two \textit{LHY} homologs in \textit{Populus} share the role that \textit{LHY/CCA1} have in other plants (Takata et al. 2009). Both \textit{LHY1} and \textit{LHY2} are functional in \textit{Populus}, although \textit{LHY2} show roughly five-fold higher expression than \textit{LHY1} in \textit{P. nigra} (Takata et al. 2009). The \textit{Populus} \textit{LHY1} paralog also has a mutation at a highly conserved Ser residue located upstream of the Myb DNA-binding domain of \textit{LHY1} which is likely involved in controlling protein phosphorylation (Takata et al. 2009). The loss of such a site has been hypothesized to contribute to altered functions of a protein and all together this suggest that \textit{LHY2} is the primary \textit{LHY} gene functioning in the circadian clock of \textit{Populus} (Takata et al. 2009). However, both \textit{LHY} paralogs retain a diurnal expression pattern it is therefore not clear whether the function of \textit{LHY1} is redundant to or distinct from that of...
Why both \textit{LHY} homologs in \textit{P. tremula} appear to be targets of natural selection in \textit{P. tremula} is not clear at present. A more detailed functional characterization of the two \textit{LHY} homologs is clearly needed to evaluate what possible function the two \textit{LHY} mutations have in controlling natural variation in growth cessation in \textit{Populus}.

\textbf{Conclusions}

Most traits that influence fitness in natural populations are quantitative in nature and are hence controlled by many genes. Furthermore, these genes often interact in regulatory networks, and although most of these genes are potentially important in controlling the expression of a given trait, only a small fraction of these genes actually have ecological relevant variation segregating in natural populations. For instance, over 30 genes have been shown to be involved in regulating flowering time in \textit{Arabidopsis}, and crossing experiments have identified at least 14 unique QTLs (Koornneef et al. 2004). Nevertheless, two genes, \textit{FRI} and \textit{FLC}, explain over 70\% of the natural variation in flowering time among \textit{Arabidopsis} accessions collected from the wild (Koornneef et al. 2004, although see Scarcelli et al. 2007 for a different view).

In this paper we have shown that segregating variation in only three genes from the photoperiod pathway explain a significant proportion of naturally occurring variation in growth cessation, a highly adaptive trait in \textit{P. tremula}. We also show that three of these SNPs can be identified as targets of spatially variable selection as indicated by the presence of allele frequency clines across a latitudinal gradient. However, none of these loci are identified as target of diversifying selection based on \textit{F}\textsubscript{ST}-outlier approaches. These seemingly contradictory observations actually fit theoretical predictions which show that even fairly strong diversifying selection on a quantitative trait will only have relatively minor effects on allele frequencies at the underlying QTLs (Latta 1998, Le Corre and Kremer 2003). Our analyses also show that even if selection at individual QTLs is weak enough, relative to migration, to not identify individual locus as outliers in \textit{F}\textsubscript{ST}-based tests, selection is still strong enough to generate substantial heterogeneity in genetic
differentiation among loci. This suggests that only searching for outlier loci, showing enhanced
levels of genetic differentiation, can miss a large number of potential loci contributing to local
adaptation, especially in species experiencing high levels of gene flow.

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**Figure legends**

**Figure 1.** Plots of derived allele frequency clines for four SNPs showing clinal variation. The left column show population allele frequencies versus latitude and the right column show population allele frequencies versus population means for growth cessation (in days). Note that populations are arranged from south to north along the x-axis in both figures to allow for easy comparison between figures. Dotted line in each figure is the least square regression line of mean allele frequency versus either latitude or population mean of growth cessation.

**Figure 2.** A) Analysis of genetic differentiation using the method of Foll and Gaggiotti (2008). Estimates of $F_{ST}$ for photoperiod (black circles) and reference SNPs (white circles) are plotted against empirical $p$-values for each SNP. The vertical dashed line denotes a posterior probability for an outlier caused by natural selection of 86% (corresponding to a $\log_{10}$(Bayes Factor) of 0.79). SNPs showing significant allele frequency clines are indicated by arrows. B) Distribution of heterozygosities for photoperiod and control SNPs. The data points have been jittered horizontally to increase visibility of individual data points. C). The distribution of the ratio of among-locus heterogeneity for photoperiod and control SNPs ($k_{\text{photo}}/k_{\text{control}}$) 10⁴ samples where SNPs were randomly assigned to the two groups. The resulting distribution of $k$ was compared to the value of $k$ observed in the data (arrow).

**Figure 3.** Quantile-quantile plots of the distributions of covariance of allelic effects for pairs of control SNPs or photoperiod SNPs against simulated data. Data for the control SNPs show a good correspondence with the simulated data whereas the photoperiod SNPs show an excess of pairs with unusually large positive or negative covariances of allelic effects.
Figure 4. Box plots of genotypic means of days until growth cessation for SNPs from A) PHYBE S383T, B) PHYB2 T608N, C) PHYB2 L1078P, D) LHY1 (syn1466) and E) LHY2 (S681N). Means are calculated from the residuals after removing the effects of fixed effects of site, year and block.
Figure 1.

[Graphs showing frequency of events against latitude and days until growth cessation for PHYB2 1823, PHYB2 6147, LHY1 1466, and LHY2 2327.]
Figure 2.
Figure 3.
Figure 4.
Table 1. Pairs of SNPs showing the highest covariance of allelic effects for growth cessation.

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<sup>1</sup>Probability of clinal variation of allele frequency with latitude

<sup>2</sup>Covariance of allelic effects (calculated using Equation [2])
Table 2. Significant associations with growth cessation for SNPs in the photoperiodic pathway.

Only SNPs showing significant associations after correction for multiple testing using the false discovery rate (FDR) are shown.

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<tr>
<th>SNP</th>
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<th>q</th>
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<th>R²(^2)</th>
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**All significant SNPs**

0.325

**All significant SNPs and interactions**

0.423

\(^1\)p-value controlling for effects of other significant SNPs

\(^2\)Percent variation explained
### Supplementary Table 1.

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